REVIEW ARTICLE

Human gene therapy comes of age

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Advances in the understanding of molecular biology of human disease and the development of efficient gene transfer techniques have resulted in practical approaches to human gene therapy, with new techniques being developed at an increasing rate. The first trials have now begun in humans and initial results are positive.

THE treatment of human disease by gene transfer has now moved from the theoretical to the practical realm. The first human gene therapy trial was begun in September 1990 and involved transfer of the adenosine deaminase (ADA) gene into lymphocytes of a patient having an otherwise lethal defect in this enzyme, which produces immune deficiency. The results of this initial trial have been very encouraging, and have helped to stimulate further clinical trials.

Many assumptions about the nature of initial human gene therapy trials have proved inaccurate. The first approved trial of gene transfer into humans in May 1989 did not involve gene therapy at all, but was performed to track tumour-infiltrating lymphocytes after infusion into patients with melanoma. Many had assumed that haematopoietic stem cells would be the first target of gene therapy because of the long-term persistence of these cells and the variety of diseases that affect the many cell lineages that are derived from stem cells. But difficulties with efficient gene transfer and appropriate gene expression in this system have slowed this approach. Instead, work has focused on more differentiated somatic cells, as evidenced by an approved human gene therapy trial involving the transfer of low-density lipoprotein (LDL) receptor to hepatocytes. Finally, gene therapy may have more immediate application to acquired diseases, such as cancer or infectious disease, rather than the single-gene genetic defects originally proposed as primary targets. An example is a clinical trial of the use of cytokine gene transfer into tumour cells to stimulate host immune response against these tumour cells.

Here I will summarize the present status of human gene therapy trials, describe exciting new results that may soon lead to human trials, and attempt to identify areas of research that will be important for the evolution of human gene therapy. In part, this review has grown out of a meeting at the National Institutes of Health in Bethesda in December 1991, where the explosion of new ideas and major advances was very apparent.

Human gene therapy approval process

Proposals for human gene therapy in the United States must pass several levels of review. There is much overlap in the purview of the numerous committees, but given the novelty of human gene therapy, this redundancy is justified. The principal issues that are addressed are the safety of the procedure for both the patient and the general public, the anticipated benefit to the patient in comparison to the potential risk, and in the case of marking experiments where the patient probably will not benefit directly from the procedure, the likelihood that useful information will be derived from the clinical trial. These issues are detailed in the guidelines for human gene transfer[1] adopted by the Recombinant DNA Advisory Committee (RAC). The Food and Drug Administration (FDA) has adopted its own set of guidelines[2] which primarily deal with the characteristics, production and certification of the biological materials used for gene transfer. Table 1 lists the human gene transfer protocols that have been approved by the RAC and all of its subcommittees. Some of these protocols have not received final approval from the FDA, but this should be regarded as more of a technical than a conceptual hurdle.

Approved human gene marking trials

All of the cell-marking protocols approved so far (Table 1) use retroviral vectors to transfer marker genes into cultured human cells that will be returned to the same patient from whom the cells were obtained. The first class of marking experiments involves the use of the marker to follow cells having presumed therapeutic use in patients. One such cell type, tumour-infiltrating lymphocytes, can be recovered from tumours, grown *in vitro*, and infused in large numbers into patients. This procedure results in clear clinical improvements in many cancer patients, although in most cases the disease recurs[3]. Cell marking is used to study the persistence and homing to tumour sites after reinfusion. Retroviral vectors have proved safe and useful in initial experiments[4], although the information gained from these experiments has not yet led to improvements in tumour-infiltrating lymphocyte therapy.

Cell marking will be used to follow donor hepatocytes used to treat acute hepatic failure. Hepatocytes will be marked with retroviral vectors in short-term culture because hepatocytes are difficult to grow and may not engraft after prolonged culture. Because of this, it is difficult to select for the presence of the transferred drug marker, thus the number of hepatocytes that are marked is variable. But initial experiments[44] have shown that a significant number of the hepatocytes can be marked and, with polymerase chain reaction (PCR)-based techniques for detection of vector DNA, the modified hepatocytes should be detectable in liver biopsies. Such marking will allow the effectiveness of hepatocyte transplantation to be assessed.

One of the most recently approved cell marking protocols involves marking human immunodeficiency virus (HIV) antigen-specific T cells that can be grown in culture and that will be used in patients to kill HIV-infected cells. The cells will be used in the context of bone marrow transplantation for treatment of lymphoma, a common complication of AIDS. AIDS patients with non-Hodgkin's lymphoma will be given bone marrow from normal matched donors after high-dose chemotherapy and total body irradiation to eradicate lymphoma, which also destroys the patients' bone marrow cells. Normal marrow from matched donors will then be administered to replace the patients' haematolymphoid system. In an attempt to protect the incoming normal marrow from being infected by residual HIV in the patient, Zidovudine (AZT) will be administered to block virus spread by inhibiting viral reverse transcription, and HIV antigen-specific killer T cells will be administered to kill residual HIV-infected cells.

As an interesting safety feature, the marking vector in this study confers both hygromycin resistance and ganciclovir sensitivity owing to the expression of a bifunctional protein encoded by portions of the bacterial hygromycin phosphotransferase and herpes simplex virus thymidine kinase genes[5]. Thus vector-transduced cells can be positively selected in culture to ensure that the infused cells all carry the vector, and can be negatively selected in the patient by administration of ganciclovir. This

TABLE 1 Approved human gene transfer trials

Experiment type	Description	Principal investigator	US Institution
Cell marking	*neo* marker transfer to tumour-infiltrating lymphocytes	S. A. Rosenberg	National Institutes of Health (NIH)
		M. T. Lotze	University of Pittsburgh
		J. S. Economu	University of California, Los Angeles
	neo marker transfer to hepatocytes used to treat acute hepatic failure	F. D. Ledley	Baylor College of Medicine, Houston
	hph-tk marker transfer to HIV antigen-specific T cells used for treatment of AIDS	P. D. Greenberg	Fred Hutchinson Cancer Research Center, University of Washington
	neo marker transfer to leukaemic cells that may be present in autologous marrow used for treatment of leukaemia	M. K. Brenner	St Jude Children's Research Hospital, Memphis
		A. B. Deisseroth	M.D. Anderson Cancer Center, University of Texas
		K. Cornetta	Indiana University, Indianapolis
	neo marker transfer to neuroblastoma cells that may be present in autologous marrow used for treatment of neuroblastoma	M. K. Brenner	St Jude Children's Research Hospital, Memphis
Gene therapy	ADA gene transfer to lymphocytes from ADA-deficient patients	R. M. Blaese	NIH
	LDL receptor gene transfer to hepatocytes from LDL receptor-deficient patients	J. M. Wilson	University of Michigan
	Tumour necrosis factor gene transfer to tumour-infiltrating lymphocytes	S. A. Rosenberg	NIH
	Tumour necrosis factor gene transfer to tumour cells	S. A. Rosenberg	NIH
	IL-2 gene transfer to tumour cells	S. A. Rosenberg	NIH
	Antigen transfer to tumour cells *in situ* to stimulate immune rejection	G. J. Nabel	University of Michigan, Ann Arbor
	Toxin gene transfer to ovarian carcinoma cells for treatment of ovarian cancer	S. M. Freeman	University of Rochester
	ADA gene transfer to peripheral blood stem cells from ADA-deficient patients	R. M. Blaese	NIH

These trials have been approved by the Recombinant DNA Committee of the NIH as of February 12, 1992, but have not necessarily been finally approved by the US Food and Drug Administration. Applications for these trials are published regularly in *Hum. Gene Ther.*

would allow selective killing of the transduced T cells in patients if a problem arises with the cells, such as uncontrolled growth, or with too vigorous a response of the T cells against HIV-infected cells.

The second class of cell-marking protocols (Table 1) involves an attempt to detect residual cancerous (leukaemia or neuroblastoma) cells in marrow infused into patients during autologous transplantation for disease treatment. In these studies, bone marrow is collected from a patient before aggressive chemo- and radiotherapy for ablation of cancerous cells in the patient. The bone marrow is then reinfused to rescue the patient from this otherwise lethal ablative therapy. Relapse of disease in a patient may be due to residual cancerous cells in the patient or in the infused marrow. The gene-marking experiment is an attempt to resolve this question and, based on the results, to develop improved strategies for ablation of cancerous cells in the patients or for removal of these cells from the infused marrow. The difficulty in these experiments is that a very low number of cancerous cells in the infused marrow might cause recurrent disease and current techniques mark only a fraction (about 1 to 10%) of the cancerous cells present. Thus the ability to resolve the question of the source of recurrent disease is limited.

Approved gene therapy trials

All of the gene therapy trials approved so far (Table 1) use retroviral vectors for gene transfer into cultured human cells that will then be administered to patients, with the exception of a protocol that employs liposomes to transfer antigen-encoding plasmid DNA directly into tumour cells *in situ*. The first class of experiments involves the transfer of genes to treat single-gene inherited disorders, the classical targets envisioned for gene therapy. The first human gene therapy trial involves treatment of ADA deficiency by transfer of the ADA gene into patients' T-cells, the cells that are most affected by this disorder. ADA deficiency leads to high levels of 2'-deoxyadenosine in the circulation which is toxic to both T and B cells and results in severe combined immunodeficiency. ADA deficiency is a lethal disorder that can be corrected by bone marrow transplantation, but unfortunately only about one third of such patients have suitably matched donors. The disease can also be partially corrected by weekly injections of bovine ADA that has been conjugated to polyethylene glycol (PEG-ADA) to increase its half-life in the circulation. Although PEG-ADA treatment can reduce systemic 2'-deoxyadenosine levels and result in a marked disease improvement, it is becoming clear, in part from the ongoing gene therapy trial, that it is important to produce ADA within T cells for more complete correction of the disease phenotype.

In the current gene therapy trial, two ADA-deficient patients receiving PEG-ADA are also being treated by infusions of their own cells that have been transduced with a retroviral vector that expresses human ADA and neomycin as a selectable marker. The first patient has received eight infusions over 10 months. Significantly, the ADA level in circulating cells has increased from <2% to 20% of normal and the circulating lymphocyte count has gone from below normal ($570\ \mu l^{-1}$) with only PEG-ADA therapy into the normal range ($2{,}100\ \mu l^{-1}$). Immune func-

tion in both patients has improved significantly as measured by isohaemagglutinin titres, skin tests for antigen sensitivity, and cytotoxic T cell assays. Contrary to early fears that the therapy would have no long-term benefit because of a short lifespan of the introduced cells, a large number of the corrected T cells have persisted in the first patient for over 6 months after cell infusions were stopped.

The second gene therapy trial involving correction of single-gene hereditary defects is an attempt to restore LDL receptor function in patients with defective or absent LDL receptors by gene transfer into hepatocytes. Unlike the case of ADA deficiency, a suitable animal model for this disorder exists, the Watanabe hyperlipidaemic rabbit. Recent experiments showed that transfer of the rabbit LDL receptor into rabbit hepatocytes in short-term culture, followed by reintroduction of these cells into the liver, resulted in a 30% average reduction in cholesterol levels in the circulation for up to 4 months[6]. These results are very encouraging for the clinical use of these techniques in humans.

The second class of gene therapy experiments involves the transfer of genes to stimulate immunity against or otherwise cause the destruction of tumour cells. The first experiment of this type involved transfer of a tumour necrosis factor (TNF) gene to tumour-infiltrating lymphocytes, in the hope that localized secretion of TNF by these cells, which home into tumour deposits, would enhance immune destruction of tumour cells. Part of the rationale for this experiment is the observation that systemic levels of TNF that cause toxicity in humans are well below the levels that are effective against tumour cells in mouse models, and therefore if the TNF could be synthesized at high concentrations locally it might be effective while avoiding systemic toxicity.

Experiments have been initiated to transfer genes encoding TNF or interleukin-2 (IL-2) into tumour cells in patients in the hope that secretion of these cytokines will stimulate a tumour-specific immune response that would either result in tumour destruction at other sites or allow the collection of more effective TIL from lymph nodes near the site of the injected tumour cells. These experiments are an outgrowth of results from many groups that have shown anti-tumour responses in mice to tumour cells secreting cytokines, including interleukins 2 and 4, γ-interferon, granulocyte colony-stimulating factor, and tumour necrosis factor α (refs 7–14). These studies generally show that injection of cytokine-secreting cells along with parental tumour cells at the same site results in killing of both the modified and unmodified tumour cells, and that injection of cytokine-secreting cells can generate a lasting immunity against the parental unmodified tumour cells. But only one group has shown that cytokine-secreting tumour cells can be effective in stimulating destruction of previously injected parental tumour cells[14], a property that will be important for treatment of most human patients. Even so, this type of therapy might be useful for patients who are apparently disease-free as a result of other antitumour therapy, who would be treated with cytokine-secreting tumour cells in an attempt to prevent future disease recurrence. Although these studies are encouraging, it remains to be seen how general this effect is with regard to human cancer, especially given the variable results seen in mice.

The first attempt (Table 1) to transfer genes directly into somatic cells of a patient without removing the cells to perform the gene transfer procedure involves the transfer of a human class I major histocompatibility antigen (HLA-B7) into tumour cells of patients who do not express this antigen in the hope of stimulating immunity against the tumour cells. The procedure involves direct injection of liposome/plasmid DNA complexes into tumour masses. If such direct injection techniques are effective, they would provide a much simpler and less costly alternative to current *ex vivo* gene transfer methods.

Another gene therapy trial (Table 1) involves an attempt to treat ovarian cancer by intraperitoneal injection of a human ovarian carcinoma cell line carrying the herpes simplex virus (HSV) thymidine kinase gene. The cells will be lethally irradiated (10,000 rad) before injection to prevent further growth of the cells. At the same time, the patients will be treated with ganciclovir, which can be metabolized by the thymidine kinase gene to a cellular toxin. Anti-tumour effects are presumed to occur by the effect of this toxin on nearby patient ovarian cancer cells, and by stimulation of immunity against tumour cells. Only limited animal data support these concepts, and the mechanism of tumour-cell killing and relevance to treatment of ovarian cancer in humans is far from clear.

Gene transfer vectors

Retroviral vectors. So far all but one of the approved gene transfer trials in humans rely on retroviral vectors for gene transduction, thus it is important to understand the advantages and limitations of this system. Retroviral vectors in this context are retroviruses from which all viral genes have been removed or altered so that no viral proteins are made in cells infected with the vector. Viral replication functions are provided by the use of retrovirus 'packaging' cells that produce all of the viral proteins but that do not produce infectious virus. Introduction of the DNA form of a retroviral vector into packaging cells results in production of virions that carry vector RNA and can infect target cells, but no further virus spread occurs after infection. To distinguish this process from a normal virus infection where the virus continues to replicate and spread, the term transduction rather than infection is often used.

The major advantages of retroviral vectors for gene therapy are the high efficiency of gene transfer into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction. The ability to transfer genes efficiently and stably to target cells, especially primary somatic cells, is not shared by other gene transfer techniques and is the major attraction of retroviral vectors for use in gene therapy. Major disadvantages include the apparent inability of retroviral vectors to infect nondividing cells[15], and an inherent inability to characterize completely the retroviral vector preparations used for gene transduction because retroviral vectors cannot be made synthetically but must be produced by cultured cells. Unlike proteins or other simple compounds, retrovirus vectors are complex mixtures of proteins and nucleic acids and cannot be purified to homogeneity after production. This disadvantage means that vector-producing cell lines must undergo extensive testing for possible adventitious microorganisms, including replication-competent retroviruses. Other contaminants such as cellular RNAs that are packaged into retroviral vectors cannot be removed. Some of these RNAs can be reverse transcribed and integrated in cells transduced with retroviral vectors[16], and only experience in animal models and in humans will determine their possible effects.

Two other potential problems with retroviral vectors warrant discussion, those of insertional mutagenesis and potential helper virus production. Problems with insertional mutagenesis, such as activation of cellular oncogenes, are shared with any gene transfer technique that results in integration of new sequences into the cellular genome, with the possible exception of adeno-associated virus, which preferentially integrates into one region in chromosome 19 (refs 17, 18). Although there are many examples of retroviral activation of cellular oncogenes in mice, these events occur in the context of a spreading infection by replication-competent virus. Whether such events can occur at appreciable rates after infection by replication-defective retroviral vectors remains to be seen.

The potential for production of replication-competent (helper) virus during the production of retroviral vectors remains a concern, although for practical purposes this problem has been solved. In the human trials so far, none of the production batches of retroviral vectors and none of the human patients have tested

positive for helper virus (P. Tolstoshev and R. Overell, personal communications). The potential for helper virus production depends on viral sequences both in the retroviral vector and in the packaging cells used for vector production (reviewed in ref. 19). The packaging cells contain all sequences necessary for viral protein synthesis and the retroviral vector contains the sequences necessary in *cis* for virus transmission, thus recombination between these sequences has the potential to generate helper virus. Particularly important is the avoidance of homologous overlap between the separate sequences. So far, all FDA-approved vectors have been made by using PA317 amphotropic retrovirus packaging cells[20]. PA317 cells were made by transfection of mouse cells with DNA containing a contiguous sequence encoding all of the retroviral proteins. It is clear that PA317 cells can produce helper virus after introduction of a retroviral vector that has extensive homologous overlap at both ends of this sequence[21,22]. But use of vectors having little or no overlap with viral sequences in the PA317 cells eliminates helper virus production even by stringent assays that allow for amplification of such events[21,22]. Other packaging cell designs involve separation of different retroviral coding regions onto different plasmids, which should reduce the possibility of helper virus production by recombination, and vectors produced by such packaging cell lines are currently undergoing review by the Food and Drug Administration.

Recently, three out of eight rhesus monkeys transplanted with bone marrow infected with a retroviral vector preparation that was heavily contaminated with replication competent retrovirus have developed thymic lymphoma after about 6 months[23]. The vector used was produced by co-cultivation of packaging cell lines to produce a high vector titre, but helper virus was also generated by this procedure[30]. Because high vector titre improves the infection rate in bone marrow cells, the helper-contaminated vector was used in an attempt to show bone marrow stem cell infection in monkeys. Disease induction in these monkeys contrasts with previous experiments which did not detect disease in cynomologous macaque and rhesus monkeys after exposure to amphotropic helper virus[24,45], and which were used to support the safety of proposed human gene therapy experiments. The occurrence of disease after exposure of some monkeys to replication-competent retrovirus reinforces the need for stringent tests for the absence of helper virus in vector preparations for use in humans.

The other gene transfer method that has been approved for use in humans is the transfer of plasmid DNA in liposomes directly to tumour cells *in situ*. Plasmid DNA should be easy to certify for use in humans because, unlike retroviral vectors, it can be purified to homogeneity. In this case, stable integration of the DNA into transduced tumour cells is not required for therapy as transient expression may suffice.

Adenovirus vectors. Recent work with gene transfer vectors based on adenovirus suggests that this vector system will soon be an important tool in gene therapy. Major advantages of adenovirus vectors are their potential to carry large segments of DNA (36-kilobase-pair genome), very high titre (10^{11} ml^{-1}), ability to infect nonreplicating cells, and suitability for infecting tissues *in situ*, especially the lung. Major disadvantages are the inclusion of many adenovirus genes in current vectors that may stimulate immunity or have other adverse effects, and potential instability of gene expression because the vector does not integrate into chromosomal DNA. The most striking use of this vector so far is to deliver a human cystic fibrosis transmembrane conductance regulator (CFTR) gene by intratracheal instillation to airway epithelium in cotton rats[25]. Expression of the transferred CFTR gene was detected for 6 weeks after the procedure by northern analysis, and human CFTR protein was detected by using an anti-human CFTR antibody at 11 to 14 days after infection. These encouraging results suggest that human trials may soon begin. The rapid progress in cloning the gene involved in cystic fibrosis, understanding the disease biology, and the development of treatments for this disease is truly impressive.

Physical DNA transfer methods. In addition to liposome-mediated DNA transfer, which will be used to transfer antigen-encoding genes into tumours in humans, several other methods for the direct transfer of plasmid DNA into cells show promise in human gene therapy. Both involve targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins. In one method, polylysine is conjugated to an asialoglycoprotein which is then used to form a complex with plasmid DNA. After injection into animals, the complex is specifically targeted to unique receptors on hepatocytes that internalize galactose-terminal (asialo) glycoproteins. Gene expression is transient after transfer, but inclusion of a two-thirds partial hepatectomy step shortly after infusion of complexed DNA leads to hepatocyte replication and prolonged expression of the introduced gene. Using the latter technique, transfer of a gene encoding human serum albumin into Nagase analbuminaemic rats resulted in circulating human albumin levels of about 30 μg ml^{-1} for over a month[26]. More recent results show that the expression persists at this level for four months but then declines to undetectable levels by six months (G. Wu, personal communication). Although this level of albumin is 1,000 times lower than normal circulating levels of albumin, for other blood proteins this would be a quite respectable level of protein production. For example, normal levels of clotting factor IX are only 5 μg ml^{-1}. But to extrapolate these results to other serum proteins one must consider the relatively long serum half-life of albumin of about 2 weeks. Factor IX, for example, has a half-life of only 19 h, thus rate of production of factor IX must be about 20-fold higher than albumin to achieve the same plasma concentration.

Another method for physical transfer of DNA to cells involves targeting of the DNA to the transferrin receptor on cells by complexing the DNA with transferrin[27]. More recently, the method has been dramatically improved by the use of adenovirus to facilitate exit of DNA from endosomes before being targeted to lysosomes for destruction[28]. This technique allows transient high-level expression of genes in many cell types, and may be useful in providing short-term gene therapy. Refinement of this technique may involve purification of specific adenovirus proteins that could be used to facilitate physical entry of DNA in general.

Target tissues

Haematopoietic stem cells. One of the main targets for gene therapy is the haematopoietic system because of well developed procedures for bone marrow transplantation, the many types and wide distribution of haematopoietic cells, and the existence of many diseases that affect haemotopoietic cells. The target for gene transfer is the haematopoietic stem cell, or long-term repopulating cell, that is present at low frequency in bone marrow and gives rise to all myeloid and lymphoid cells over prolonged periods. Although many groups have reported transfer and long-term expression of genes in mouse haematopoietic cells[46], the same has not been achieved in larger outbred animals. Monkeys are perhaps the best animal model for procedures that will be used in humans because of their phylogenetic proximity, but dogs are also a good model and have been instrumental in the development of many bone marrow transplantation procedures.

Experiments in monkeys have documented expression of human ADA and neomycin phosphotransferase at low levels in peripheral blood cells for up to 4 months after bone marrow infection with a retroviral vector carrying ADA and *neo* genes[29]. Persistence of vector sequences in about 1% of peripheral blood cells for up to 3 months after retrovirus-mediated gene transfer has also been demonstrated using a high-titre vector carrying the *neo* gene[30], and more recent experiments from the same group document still longer persistence of vector sequences in additional animals. But these experiments are complicated by

the presence of helper virus in the vector preparations used for infection, which can lead to spread of the vector in the animal and has caused lymphoma in several animals. Thus the feasibility of gene transfer in monkeys must be re-evaluated by using helper-free vectors for gene transfer.

Early gene transfer experiments into canine haematopoietic cells resulted in only transient presence of vector function in haematopoietic cells[31]. Recent improvements in the vector infection protocol have resulted in persistence of vector sequences in both lymphoid and myeloid cells in several dogs for over two years, indicating successful transduction of haematopoietic stem cells[32]. In this case, the vectors were helper-virus-free and helper virus has not been detected in blood from animals receiving vector-infected cells, nor have the animals developed vector-related disease. The vectors used carry the human ADA and bacterial *neo* genes, and drug resistance due to expression of the *neo* gene has been observed intermittently in up to 10% of haematopoietic colonies (CFU-GM) grown from bone marrow for two years. Although these results are encouraging, the level of *neo* expression is low and expression of the ADA gene was not detected.

Retroviral vectors that are similar to those used in monkeys have also been used to infect haematopoietic cells in cats[33]. Vector sequences and drug-resistant haematopoietic cell colonies were observed in some of these animals for over 2 years. Human ADA in cats infected with a vector that carried the ADA gene was not detected. As in the monkey experiments, all vector preparations were contaminated with helper virus, and helper virus was detected in the experimental animals. Two of the four experimental animals developed diabetes mellitus 90 days after receiving infected marrow, most probably due to the presence of helper virus.

In utero transfer of the *neo* gene into haematopoietic cells of fetal sheep has been accomplished[34] by removing circulating cells 7 weeks before birth, infecting these cells *in vitro*, and returning the infected cells to the fetus. Persistence of the infected cells after birth was demonstrated by detection of *neo* sequences in blood, bone marrow, spleen and thymus samples from some animals 1 week after birth, detection of neomycin phosphotransferase in bone marrow of one animal 6 weeks after birth, and by the presence of G418-resistant haematopoietic colony-forming cells in marrow for more than 2 years after birth. The vectors used were the same as those used in the above experiments in monkeys and cats and were contaminated with helper virus. The same infection procedure was not successful with marrow cells from adult sheep instead of fetal cells. This difference is most probably due to the high replication rate of fetal haematopoietic cells, which would make them more susceptible to infection by the retroviral vector.

It is clear from the above studies that more needs to be done to improve the efficiency of gene transfer and expression in large animal models to provide a solid rationale for extending these studies to trials of gene transfer into human haematopoietic stem cells. Alternatively, some researchers are attempting to identify protocols in which the ability to infect human stem cells can be tested directly without undue danger to the patient. For example, in experiments designed to mark cancerous cells in bone marrow to study the source of cancerous cells during relapse, it is possible that bone marrow stem cells will be marked as an incidental result of the procedure. The presence of the vector in a variety of haematopoietic lineages for long periods after the procedure would provide evidence of stem cell infection. The difficulty with this approach is that conditions required for efficient gene transfer into haematopoietic stem cells are likely to be very different from those required for efficient transfer into cancerous cells in marrow.

Another attempt to infect human stem cells has been approved as an amendment to the protocol involving ADA gene transfer to lymphocytes from ADA-deficient patients. I have listed this trial as a separate trial in Table 1 because it represents a conceptually different experiment from the first trial for several reasons. Most importantly, modified lymphocytes can be grown in cell culture and gene transfer verified before the cells are returned to the patient, whereas no *in vitro* assays exist for human haematopoietic stem cells, let alone for the rate of gene transduction. In this trial, patients will be pretreated for about a week with G-CSF in an attempt to stimulate the circulation of stem cells that are normally present in bone marrow. Circulating mononuclear cells will be obtained by apheresis and enriched for stem cells by selection for the cell surface marker CD34. The enriched cells will be incubated for a few days with virus in a cocktail of growth factors that is expected to stimulate stem cell replication, and will then be reinfused. Monkeys provide a good model for this experiment because several antibodies against human CD34 also react with monkey CD34, and although there are no ADA-deficient monkeys, the overall strategy of the experiment could be tested. However, the experiment was approved in the absence of compelling animal data, presumably because retroviral transduction of $CD34^+$ cells should present little additional danger to a patient who is already receiving large numbers of retrovirus-transduced lymphocytes, and there might be some benefit if it works.

Muscle cells. It has been possible to transfer genes directly into skeletal muscle *in vivo* by direct injection of DNA[35,36]. Injection of a plasmid containing a β-galactosidase gene into mouse skeletal muscle resulted in β-galactosidase expression in individual myotubes in the area of the injection. Injection of a plasmid containing a luciferase gene resulted in expression of luciferase for 2 months at relatively constant levels, suggesting that expression may persist for longer periods. The injected DNA seemed to persist in an unintegrated extrachromosomal state. Attempts to transfer genes to other tissues by using DNA have not been successful, suggesting that the ability to incorporate and express naked DNA is unique to muscle[35]. Unfortunately, transfer of the luciferase gene into skeletal muscle of monkeys resulted in much lower levels of luciferase expression than that observed in mice[37]. It will be interesting to see if this technique can be developed to provide long-term gene expression at useful levels, as the technique would provide an exciting alternative to the more complex methods for gene transfer currently in use.

Recent experiments have demonstrated that genetically modified myoblasts can be injected into skeletal muscle, fuse with existing muscle and continue to express the transferred gene[38,39]. After transfer of genes encoding human growth hormone, growth hormone was detected in the serum of recipient animals at levels from 1 to 16 ng ml^{-1} for up to 85 days. But the C2C12 myoblast cell line used in these experiments is an immortal mouse myoblast line that can form tumours in recipient animals. Indeed, the level of growth hormone detected in the longest experiments increased with time, suggesting that the implanted cells were continuing to grow[39]. These results are reminiscent of early studies with immortal mouse fibroblast cell lines (NIH 3T3 cells) that were modified to express human factor IX. After subcutaneous or intraperitoneal injection, the cells continued to grow and circulating levels of human factor IX reached 500 ng ml^{-1} plasma[40]. Normal fibroblasts behave quite differently and can suppress expression of transferred genes after implantation[41]. Thus it will be interesting to see if normal myoblasts continue to express introduced genes at meaningful levels after implantation, and whether these techniques can be applied to large animals.

In addition to skeletal muscle myoblasts, smooth muscle cells present in blood vessels can also be cultured, genetically modified and returned to blood vessels after disruption of the vessel wall[42]. Normal rat smooth muscle cells were transduced with a retroviral vector carrying human ADA and bacterial *neo* genes and were returned to the arterial wall after denudation of endothelial cells by passage of a balloon catheter. Human ADA was expressed in the vessel wall for at least 6 months at

TABLE 2 Diseases caused by single gene defects: current targets for gene therapy

Disease	Defective gene
Immunodeficiency	Adenosine deaminase
	Purine nucleoside phosphorylase
Hypercholesterolaemia	LDL receptor
Haemophilia	Factor IX
	Factor VIII
Gaucher's disease	Glucocerebrosidase
Mucopolysaccharidosis	β-glucuronidase
Emphysema	α1-antitrypsin
Cystic fibrosis	Cystic fibrosis transmembrane regulator
Phenylketonuria	Phenylalanine hydroxylase
Hyperammonaemia	Ornithine transcarbamylase
Citrullinaemia	Arginosuccinate synthetase
Muscular dystrophy	Dystrophin
Thalassaemia	β-globin
Sickle cell anaemia	β-globin
Leukocyte adhesion deficiency	CD-18

This list indicates the principal current targets for gene therapy efforts and is not meant to be complete. Many of the diseases listed can be caused by defects in more than one gene; the gene defect listed is the defect targeted by current research.

relatively constant levels that were similar to the endogenous rat ADA levels in the vessel.

Disease targets

Research in gene therapy has focused on a variety of diseases (Table 2), most of which involve recessive single-gene disorders that can be corrected by addition of a functioning gene to the appropriate cells. In cases like the clotting factor deficiencies, almost any somatic cell can be a target for gene therapy because the gene product is secreted and is required systemically. In other cases the gene must be delivered to a specific cell type, such as the hepatocyte in the case of the urea cycle enzyme ornithine transcarbamylase.

An exciting application of gene therapy will be the treatment of acquired diseases such as cancer and infectious disease. As discussed above, trials for cancer treatment by cytokine gene transfer have already begun. Recent results indicate that AIDS may also be an early target for gene therapy. The focus of such an approach would be to use gene transfer to protect cells from infection by HIV or to prevent the production of HIV from a cell that is already infected. Of the ways that can be imagined to achieve this end, an approach using 'TAR decoys' looks very promising[43]. In this approach, copies of the short region of HIV that is responsive to the viral transactivator (*tat*) protein, called the transactivation response (TAR) element, are overproduced by including the TAR element in a transfer RNA transcription unit. Transcription by RNA polymerase III leads to very high levels of the transcripts, which presumably act to bind the *tat* protein and reduce its ability to activate HIV. Cells expressing the TAR decoys could be infected by HIV but would make only low levels of HIV proteins or virus.

Future directions

I have tried to summarize approaches that have the best potential for application to human gene therapy. Many other gene transfer techniques and target tissues are available, and we are likely to see the development of important new techniques in the future. Several areas of research will be instrumental in further progress.

A better understanding of somatic cell transplantation and the biology of the cells that contribute to reconstitution will be necessary. For example, if human haematopoietic stem cells could be expanded in number in culture without diminution of their ability to repopulate the haematopoietic system, as is possible in mice to some extent[47], such a development would revolutionize the treatment of disease. Then individual stem cell clones could be genetically modified (possibly by homologous recombination), expanded, and used for transplantation. This would be analogous to current techniques for genetic manipulation of embryonic stem cells that can reconstitute whole animals.

The development of improved gene transfer techniques, especially for *in situ* gene delivery, are clearly important. A major problem with most techniques is the inability to stably transfer genes to non-dividing cells. For example, although the elegant technique for targeting DNA to liver by complexation with asialoglycoproteins can result in efficient gene transfer *in vivo*, expression is short lived without a two-thirds hepatectomy to stimulate cell division and (presumably) genomic integration of the DNA.

The consequences of introduction of cells that in general produce proteins that are foreign to the host must be better understood, and methods to control immune response will need development. For example, production of normal LDL receptors in hepatocytes of patients that were previously receptor negative might generate a strong immune response against this new antigen. Even intracellular proteins may elicit detrimental immune responses.

In addition, we must have a better understanding of the factors that control expression of genes introduced into somatic cells. In my own work, I have observed a dramatic decrease in expression (>1,500-fold) of a retroviral vector in skin fibroblasts after transplantation, but not in culture[41], whereas the same vector promotes long-term protein production in smooth muscle cells in the same animal model[42]. The enthusiastic climate surrounding gene therapy should lead to more concentrated efforts in all of these important areas of research. □

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